58 PLANT REGENERATION IN TISSUE CULTURES OF MAIZE

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C. E. Green and C. A. Rhodes
Department of Agronomy and Plant Genetics
University of Minnesota
St. Paul, MN 55108

Tissue cultures capable of plant regeneration may be efficiently initiated from immature embryos of maize and more recently from several other sporophytic tissues including immature tassels and ears (Green and Phillips, 1975; Freeling et al. 1976; Green 1977; Molnar et al. 1980; Rhodes et al. 1982). The morphology of these cultures is typically one of a complex association of shoot and root meristems as well as less differentiated tissues (Springer et al. 1979). This morphology has been diagnostic of the capacity to regenerate plants of maize with the exception of a new culture type to be discussed below.

The various sporophytic tissues which can be used to initiate regenerable tissue cultures have their individual experimental advantages. Immature embryos are available in large numbers from a single pollination and they develop in relative synchrony on the same ear. Consequently they can be used effectively to compare genetic differences or experimental treatments among sibs. Immature tassels, on the other hand, are obtained from donor plants which are usually 6 to 7-weeks-old. This allows time for the expression of certain genetic markers which permit the identification of cytogenetically altered plants, such as haploids, monosomics, or plants with deletions, prior to initiation of cultures.

Tissue Culture Initiation

To initiate cultures from immature embryos, the husked intact ear is sterilized in 2.5% sodium hypochlorite plus a small amount of detergent for 20 min. and then rinsed three times in sterile water. Each rinse should be for a minimum of 5 minutes. Using sterile instruments in a sterile environment, such as a laminar air flow hood, the crown of each kernel is cut off and a rounded-tip spatula is used to scoop out the endosperm. Embryos up to 2 mm in length usually remain associated with the endosperm and can be seen with the unaided eye and removed from the endosperm with the tip of the spatula. The embryo is placed on the culture medium with the embryonic axis against the medium. This embryo orientation permits extensive proliferation in the scutellum and minimizes germination and further development of the embryonic axis. Up to ten embryos fit easily in a 100 x 25 mm Petri dish containing 50 ml of medium. Masking tape or Parafilm strips provide a good seal for the dishes.

Immature tassels for tissue culture studies are typically obtained from 6 to 7-week-old field or greenhouse grown plants. The tassels in these plants are usually 2 to 3 cm in length and are encased in many layers of young leaves. Sterilization of the tassel is not necessary if the leaves surrounding it are carefully removed in a sterile environment. The entire tassel is cut into 1 to 2 mm thick cross sections and all these pieces are immediately transferred to a Petri dish containing 50 ml of culture medium to minimize desiccation. Orientation of the pieces on the medium does not appear to be critical.

Although several growth medium formulations will support the growth of regenerable tissue cultures of maize, the most frequently used media are MS (Murashige and Skoog, 1962) and N6 (Chu et al. 1975, Chu 1978). A particularly critical ingredient in the medium is the auxin which must be of sufficient potency and concentration to suppress germination of immature embryos and root formation in immature tassel sections during the initiation of the tissue cultures. 2,4-dichlorophenoxy acetic acid (2,4-D) at 0.5 to 1 mg/l has been used with the greatest success as an auxin for tissue culture propagation in maize.

After three to four weeks of incubation at 25-28°C under low fluorescent light (1.5 W/m^2), regenerable cultures from immature embryos or tassels can be identified by the presence of scutellar-like bodies. Vigorous cultures will be light green or yellow and may have small leaves developing among the scutellar-like bodies. The growth and regenerability of these cultures can be maintained for several years by transferring pieces about 5 mm in diameter to fresh medium every 3 to 4 weeks.

Plant Regeneration

To regenerate plants from these cultures the auxin (2,4-D) must be greatly lowered or omitted from the medium. Depending on the degree of organization in the culture it may be useful to lower the auxin in steps via successive transfers or to remove it entirely in one transfer. Shoots which emerge are transferred to 250 ml Erlenmeyer flasks containing 75 ml of medium without 2,4-D and incubated under bright fluorescent light (8-10 W/m^2; 16/8 hr photoperiod). When an adequate root system has developed, the plant is transferred to 2 inch plastic pots containing a 1:1 mixture of sterile soil and vermiculite. It is critical at this stage to thoroughly rinse all the growth medium off the roots before transplanting the plant into soil. The plants should be kept in a humid, brightly-lighted environment for 2 to 4 weeks until established, before transplanting to a greenhouse or field. Regenerated plants often have more vigor and are more likely to produce morphologically normal tassels and ears if the various steps in regeneration and establishment are carried out in as short a time as possible. Pollination of regenerated plants is accomplished by selfing or crossing with other plants to produce the desired kernels and progeny. Seeds from regenerated plants are generally grown into plants using standard cultivation practices.

Genetic Analysis

Cytogenetic analysis of plants regenerated from diploid maize cultures, eight-months-old or less, has indicated a high degree of chromosomal stability (Green et al. 1977; McCoy and Phillips, 1982). The instability which does occur usually involves missing or broken chromosomes. Abnormal plants may have sectored tassels such that one or more of the tassel branches exhibit partial pollen sterility and one or more of the branches have normal fertility. Meiotic analysis of pollen mother cells in young tassels has identified a total of 9 cytologically abnormal plants among 277 examined (Green et al. 1977; Edallo et al. 1981; McCoy and Phillips, 1982). The limited data available on the chromosome constitution of plants regenerated from older tissue cultures indicates a much higher frequency of abnormalities. Eleven plants regenerated from three-year-old cultures all possessed the same phenotype (i.e. oppositely arranged leaves and ca. 100 cm

tall) at maturity (Green et al. 1977). Three plants were analyzed cytologically and each plant contained a broken chromosome 6, deficient for the distal third of the long arm. This is an indication that cytological abnormalities may be increased in older cultures.

An interesting aspect of recent studies is that tissue culturing induces considerable variation in the genetic makeup of cells and that this variability is recovered in the progeny of regenerated plants. A high frequency of spontaneous mutations with simple Mendelian inheritance has been observed in R2 progenies (Edallo et al. 1981). The type of endosperm and seedling mutants found were similar to spontaneous mutants described in maize. A high rate of spontaneous mutation makes these cultures of particular interest in selection experiments.

Mutants have been selected from maize tissue cultures in two separate experiments. Selection for resistance to *H. maydis* race T pathotoxin in diploid, Texas male sterile cultures resulted in the recovery of resistance which was transmitted to regenerated plants and progeny (Gengenbach et al. 1977). This selection also changed male sterility to fertility. Both resistance to the disease and fertility were inherited as maternal traits. Selection for resistance to lysine plus threonine in diploid cultures also produced resistance which was recovered in the progeny of regenerated plants (Hibberd and Green, 1982). This lysine plus threonine mutant, Ltr*-19, overproduces threonine in large quantities in the seed and is inherited as a dominant gene.

Somatic Embryogenesis

A major new type of maize tissue culture has been developed recently which is distinguished from those discussed previously by its friability, rapid growth, and capacity to regenerate plants by somatic embryogenesis (Green, 1982). These cultures are light yellow and appear undifferentiated in that little organization is visible to the eye. Closer examination by light and scanning electron microscopy, however, reveals somatic embryos which follow very closely the known developmental sequence for zygotic embryos (Randolph, 1936).

These embryogenic cultures are initiated directly from the scutellum of immature embryos or as spontaneous sectors growing from established organogenic tissue cultures of the type discussed earlier in this paper. They can be initiated and maintained on both MS and N6 medium containing 0.5 to 1.0 mg/l 2,4-D. The cultures are first noticed as small friable regions which are light yellow. These cultures may not show visible organization initially but frequently within 2 weeks of their appearance, microscopic examination reveals embryos at globular or slightly more advanced stages of development. Once established these cultures exhibit a rapid growth rate and must be subcultured every 2 weeks. They are maintained by transferring 4 to 5 pieces of callus, each 5 to 10 mm in diameter, to 100 x 25 mm Petri dishes containing 50 ml of fresh medium.

The established cultures exhibit a high degree of embryogenic activity; frequently hundreds of embryos per callus are visible. They are first seen as small globular structures and their continued development is evidenced by differentiation of the suspensor, scutellum and embryonic axis tissues. Embryo development up to the coleoptilar stage (Abbe and Stein, 1954) progresses quite normally on either MS or N6 media containing 2% sucrose

and 0.5 to 1.0 mg/l 2,4-D. Development beyond this stage is very abnormal unless the callus is transferred to medium lacking 2,4-D but with 6% sucrose to increase the osmolarity of the medium. Maturation proceeds rapidly on this medium and after 10 to 14 days embryos have developed which are similar in size to those found in seeds. Their most prominent features are a well-developed scutellum and embryonic axis. These embryos germinate rapidly when transferred to medium lacking hormones and 2% sucrose. Shoot and root development occurs simultaneously and the young plants are then grown under conditions described in the Plant Regeneration section.

These friable embryogenic callus cultures are appropriate for the initiation of suspension cultures which are grown in liquid MS medium containing 2,4-D and aerated on a gyrotory shaker. The resulting cultures are well-dispersed and composed of cell aggregates ranging from about 2 mm in diameter down to single cells. Embryo development rarely proceeds as far as the globular stage in these cultures. When these suspensions are plated on agar-solidifed MS medium, active embryogenesis is observed in the resulting callus cultures.

Other Important Factors

The genotype of the donor tissue substantially influences the ease with which tissue cultures of maize are obtained and the duration of regenerability. When using immature embryos, some genotypes (i.e. A188, WF9, and ND203) produce regenerable cultures very efficiently while similar cultures are initiated with great difficulty from other genotypes, such as W23 and A632. Regenerable cultures have been initiated from immature embryos of 70% of the approximately 40 different genotypes we have examined, without modification of the standard cultural regime. Modifications in the growth medium have improved the performance of some more difficult genotypes. Typical modifications include adjustments in the 2,4-D concentration and alternative macro-and micro-nutrient formulations of the medium. Characteristic differences among genotypes are also noted when cultures are initiated from immature tassels. Growth conditions can and should be optimized to produce the most desirable cultures from specific genotypes.

The developmental stage of the donor tissue is an important factor in the initiation of regenerable tissue cultures. As immature embryos become more fully developed, they rapidly lose the ability to initiate regenerable cultures (Green, 1977). Embryos 3 mm or longer are generally ineffective for culture initiation. For most genotypes, embryos which are 1 to 2 mm long most reliably produce the desired cultures. Immature tassels more frequently produce regenerable cultures when they are 1 to 3 cm long.

Incubation conditions used for maize tissue cultures are very similar to those for cultures of other species. The growth rate of maize cultures increases with temperature up to 30°C. The most frequently used temperature for incubation is 28°C. Temperature control in growth rooms and chambers should be as uniform as possible to minimize the condensation of water from the growth medium onto the lids and walls of Petri dishes and other containers. The greater the fluctuation in temperature, the greater the problem of condensation. Severe condensation can interfere with culture growth as well as increase the chances of microbial contamination. Humidity control in growth rooms and incubators is generally not necessary if dishes and flasks are sealed properly with tape, parafilm, or aluminum foil. These

seals are also important in preventing microbial contamination of the cultures.

Although not dependent on light, growth of the cultures is often improved and regeneration yields more vigorous plants when they are grown in the light. Typical light sources include standard Cool-White, Grow-Lux, or Agro-Lite fluorescent fixures. These provide light intensities up to 1.5 W/m^2 which is more than adequate for the cultures. Typical photoperiods are 12-16 hours of light per day.

Cultures should be subcultured as frequently as necessary to maintain vigorous growth. The actual time interval is influenced by the type of culture, volume of culture medium, and the amount of inoculum used for the subculture. Cultures with rapid growth rates, such as the friable embryogenic lines, are transferred every two weeks while slower growing cultures are transferred about every four weeks. Careful management of the subculture and incubation conditions is especially important for the maintenance of the regenerability of cultures. The potential to regenerate numerous plants from tissue cultures of maize can be maintained for several years if necessary.

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